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Effect of freeze-dried coffee solution in a high-fat diet-induced obesity model in rats: biochemical and inflammatory impacts and effects on gut microbiota

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Effect of freeze-dried coffee solution in a high-fat diet-induced obesity model in rats: biochemical and inflammatory impacts and effects on gut microbiota

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Abstract

Gut microbiota has been linked to the etiology of the obesity process. Foods that are rich in polyphenols seem to positively affect gut microbiota, and consequently prevent obesity and its comorbidities. This study investigates the effect of freeze-dried coffee solution (FCS) consumption on physiological parameters, lipid profile, and microbiota during obesity induction in rats by a high-fat diet. Animals divided into the following 56-day treatment groups: control (CT-); control + FCS (CT+); high-fat diet (HF-); and high-fat diet + FCS (HF+). The FCS combined with a high-fat diet increased fecal and cecal *Bifidobacterium* populations, decreased cecal *E. coli* population and intestinal Il1b mRNA levels. Independently of the diet type, the FCS increased serum HDL-c and did not affect body weight, food intake, LDL, triglycerides, fecal bile acids, and intestinal Il6 mRNA levels. The high-fat diet increased weight gain, hepatic cholesterol and triglycerides, fecal bile acids, fecal and cecal *Lactobacillus* populations, and it reduced food intake, fecal *E coli* population, and intestinal Il6 mRNA levels. The results suggest that consumption of FCS has a positive effect on health in rats fed with a high-fat diet by increasing *Bifidobacterium* populations and HDL-C reverse cholesterol transport to tissues, and reducing Il1b mRNA levels. However, the FCS administered in a dose of 0.39g/100g diet over a period of 8 weeks was not effective in controlling food intake and consequently preventing weight gain in the rats during obesity induction by the consumption of a high-fat diet.

Introduction

Obesity is a metabolic disease characterized by excessive fat accumulation and low-grade chronic inflammation, caused by an imbalance between food intake and energy expenditure [1,2]. Obesity is a complex process that involves genetic aspects and environmental factors (diet, lifestyle, etc.). It is considered one of the important risk factors for other metabolic diseases, such as insulin resistance, type 2 diabetes, dyslipidemias, fatty liver disease, atherosclerosis, hypertension, and stroke [2–4]. According to the World Health Organization [5], 39% of adults are overweight and 13% are obese worldwide.

Gut microbiota has been linked to the etiology of the obesity process. It can contribute to the host's insulin resistance, the chronic inflammation process, a higher energy harvest from the diet, and body fat deposition through different mechanisms of interaction with the host [3,5,6]. According to Zhao [7], the link between intestinal microbiota and the development of obesity and other metabolic diseases in hosts may be related to the toxic metabolites derived from the gut microbiota.

Evidence suggests that obese individuals have a larger population of the phylum Firmicutes in the gut compared to the phylum Bacterioidetes [6,8] and a lower diversity of intestinal microbiota populations in relation to specific genera of microorganisms [9,10]. Such an imbalance in intestinal microbiota composition leads to individuals developing obesity [6,8].

Different dietary components affect gut microbiota and may consequently promote or prevent obesity and its comorbidities. The consumption of a high-fat diet, particularly rich in saturated fatty acids, increases the proportion of Firmicutes while decreasing Bacteroidetes; it decreases gut microbiota diversity and modifies the bacterial metabolites released [11–13]. Such changes can influence satiety and fat

deposits and increase the secretion of bile acid and pro-inflammatory cytokines, leading to the subsequent development of systemic inflammation, obesity, insulin resistance, and type 2 diabetes [2,11,14,15].

Contrary to what is observed for excessive fat consumption, polyphenol-rich diets have been shown to positively modulate the gut microbiota composition and prevent obesity induced by a high-fat diet [16]. Coffee is an example of a food that is rich in polyphenols and its health effect on obese individuals has been the subject of research [17–19].

Coffee is a complex mixture of chemical compounds and its composition varies according to the species of the coffee bean (*Coffeea arabica* and *Coffea canephora* var. *Robusta*), roasting process, and fermentation process. Polyphenols (chlorogenic acids, alkaloids polyphenols, caffeine, and trigonelline) and melanoidins are considered to be the components of coffee that are most likely to promote health benefits, as 95% of these compounds reach the large intestine and therefore interact with the intestinal microbiota [18,20].

Studies that evaluate the benefits of coffee in rats fed with a high-fat diet have been carried out using coffee extracts, instant coffee, and purified bioactive components of coffee. To date, there are no studies that have evaluated the effect of incorporating coffee into food with the aim of preventing obesity in rats. Freeze drying is considered to be the best method for preserving the chemical properties of foods. Freeze-dried coffee solutions can be seen as a new product with the potential to be incorporated into different foods. Therefore, the aim of this study was to investigate the effect of a freeze-dried coffee solution when mixed with a high-fat diet on the physiological parameters, lipid profile, and microbiota of rats in a high-fat diet-induced obesity model.

2. Materials and methods

2.1 Preparation of freeze-dried coffee solution

A 10% grounded coffee solution, consisting of a blend of grains from the *Coffea arabica* species (Ponto Aralto, Jundiaí, SP, Brazil), was prepared by filtration using 100% cellulose filter paper (Original, n° 103, *Melitta*®, São Paulo, SP, Brazil) and water at 90°C. The coffee solution was freeze-dried using an industrial freeze dryer (Beta 2-8 LSC PLUS Martin Christ, Nova Analítica Ltda, São Paulo, SP, Brazil) and stored at -80ºC until the formulation of the diets. The freeze-dried coffee solution (FCS) was added to the diets at a proportion of 3.9g/kg of diet. The amount of FCS added to the rats' diet is equivalent to 163 ml/day or 1.5 cups/day of coffee, the usual mean coffee intake of the Brazilian population [21]. A previously characterization of the commercial brand *Coffea arabica* species (Ponto Aralto, Jundiaí, SP, Brazil) found 19.93, 4.22, 5.14 and 9.63 mg of caffeine fractions (3-, 4-, 5- caffeoylquinic acid)/g and 18.99 mg of total caffeoylquinic acids/g [22]

2.2. Ethics Statement

The experimental protocol was approved by the Animal Care and Use Committee of the University of Brasília, under protocol n° 25/2018, approved on 05/08/2018, in accordance with the Brazilian National Council for Animal Experimentation Control (CONCEA) and the Guide for the Care and Use of Laboratory Animals [23].

2.3 Animals

Twenty-eight male Wistar rats (Institute of Biomedical Sciences, University of São Paulo, SP, Brazil) aged 21 days old and with an average body weight of 67.37 ± 6.04g were housed individually in stainless steel cages in a room with 12/12h light/dark cycles at 22 ± 1°C. The diet was provided from 12pm to 8am, with free access to water.

The animals were kept for seven days on the AIN-93G control diet [24] for acclimatization, and then were assigned to one of the following experimental groups (six rats/group) and treated for a 56-day period with the corresponding diets: control group (**CT-**): AIN-93G diet; high-fat group (**HF-**): AIN-93G diet with 58% of fat; coffee group (**CF+**): AIN-93G diet with 3.9g of FCS/kg of diet; and high-fat + coffee group (**HF+**): AIN-93G diet with 58% of fat (51.9% of lard and 6.1% of soy oil) and 3.9 g of FCS/kg of diet. The fat percentage was determined according to the Research Diets, Inc. Diet-Induced Obesity Model (D12492, Research Diets, Inc., New Brunswick, NJ, USA).

During the experimental period, the feces of each animal were collected daily, pooled in the same tube for a week, and stored at -80ºC. This provided eight-weeks fecal samples from each animal by the end of the experimental period. These were analyzed in the first and eighth weeks of treatment. Body weight was recorded weekly and food intake daily.

At the end of the treatment period, after a 7h fasting period the animals were anesthetized with 3% isoflurane (BioChimico, Rio de Janeiro, RJ, Brazil) and euthanized by exsanguination via cardiac puncture. The large intestine (ileocecal valve to the rectum) and cecum were excised; the remaining fecal content in the large intestine and the cecum content were collected in sterile tubes and stored at -80°C. Subsequently,

the large intestine was washed in 0.9% saline solution at 4°C, immediately frozen in liquid nitrogen, and stored at -80°C for further analysis.

2.4 Lipid profile

Total cholesterol (CLT), HDL cholesterol, LDL cholesterol, and triglyceride (TG) concentrations were measured in serum and liver using commercial enzymatic/colorimetric kits (BioClin, Belo Horizonte, MG, Brazil) according to the manufacturer's assay protocol. Total lipid extraction from the liver was performed according to Vieira *et al*. [25]. Briefly, 50mg of liver was homogenized in 1ml of isopropanol and centrifuged at 2000 x *g*/10min at 4°C. The supernatant was removed and stored at -80ºC until the analysis.

2.5 Bile acid concentration in feces

An aliquot of feces from the first and eighth weeks of treatment was freeze dried at -45°C for 48h. The freeze-dried samples were macerated in a porcelain mortar using liquid nitrogen. Total bile acids were extracted in ethanol according to Kanamoto *et al*. [26] and Tamura *et al*. [27] and the concentration of total bile acids was measured according to a fluorometric method using a commercial assay kit (Sigma-Aldrich, San Luis, MO, USA), following the manufacturer's instructions.

2.6 Determination of fecal microbiota composition

2.6.1 Extraction of DNA from fecal samples and cecum content

The DNA from the feces samples was extracted using a QIAamp PowerFecal DNA kit (Qiagen, Hilden, RP, Germany) according to the manufacturer's assay

protocol, with the following modifications: 150mg of feces were used and the samples were homogenized using a cell/tissue disruptor (L-beader 6, Cotia, São Paulo, SP, Brazil) following a schedule of 2 cycles of 2500rpm/15s for cecum contents and 3 cycles of 2500rpm/15s for feces. The samples were placed on ice for 30s between each cycle.

The DNA samples were quantified by determining the absorbance at 260nm using the equation A_{260nm} x 50 x dilution factor while their purity was assessed by determining the absorbance ratios $A_{260}/A_{280\ nm}$ (~ 1.8-2.0) and $A_{260}/_{230\ nm}$ (~ 2.0) [28] and evaluating the electrophoretic DNA profile on agarose gel.

2.6.2 Real-time PCR analysis

The gut microbiota composition of *Bifidobacterium* spp., *Lactobacillus* spp., *Escherichia coli*, *Bacteroides* spp., and *Enterococcus* was evaluated using quantitative real-time polymerase chain reaction (qPCR, StepOnePlus System, Applied Biosystems, Foster City, CA, USA). Every analysis was performed in triplicate using the Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), with each well containing 2µl of sample DNA or standard, sense, and antisense oligonucleotides (Table 1) at a concentration of 100nM and water totaling 10µl. The initial DNA denaturation occurred at 95°C (20s), followed by 40 cycles of denaturation at 95°C (3s), annealing of oligonucleotides, and extension at 59-60°C (30s).

Table 1. Primers and reaction conditions for the bacterial genera analyzed by real-time polymerase chain reaction.

Microorganisms		Primers sequence (5'- 3')	T (ºC)	Reference
E. coli	F	CATGCCGCGTGTATGAAGAA	59°	[29]
	R	CGGGTAACGTCAATGAGCAAA		

***Enterococus* spp**	F R	CCCTTATTGTTAGTTGCCATCATT ACTCGTTGTACTTCCCATTGT	60°	[30]
***Bifidobacterium* spp**	F R	AGGGTTCGATTCTGGCTCAG CATCCGGCATTACCACCC	60°	[31]
***Lactobacillus* spp**	F R	TGGATGCCTTGGCACTAGGA AAATCTCCGGATCAAAGCTTACTTAT	60°	[32]
***Bacteroides* spp**	F R	GAGAGGAAGGTCCCCCAC CGCTACTTGGCTGGTTCAG	60°	[33]

F: forward primer; R: reverse primer; T: annealing temperature

Standard curves were constructed for each experiment using five sequential dilutions of bacterial genomic DNA from pure cultures ranging from 200ng to 0.064ng. The results were expressed as log10 of 16S rRNA copy number/g of feces according to the methodology described by Talarico *et al*. [34]. The different strains used were obtained from the America Type Culture Collection (ATCC) (*Escherichia coli* ATCC 25992; *Enterococcus faecalis* ATCC 19433; *Bacteroides* ATCC 25285), a commercial culture (*Bifidobacterium* spp. BL 04), and from the Tropical Cultures Collection (*Lactobacillus plantarum* UnB SBR64.1 MK5114407).

2.7 Determination of the mRNA levels of the pro-inflammatory genes associated with inflammation

2.7.1 RNA extraction and cDNA synthesis

Total RNA extraction from the large intestine was carried out using TRIzol reagent™ (Invitrogen Inc., Burlington, ON, Canada) according to the manufacturer's assay protocol. Total sample RNA was quantified by measuring the absorbance at 260nm (A_{260nm} x 40 x dilution factor) and its purity was evaluated by the absorbance

ratios A_{260nm}/A_{280nm} and A_{260nm}/A_{230nm} [28]. The integrity of the RNA bands was also verified by their electrophoretic profile on agarose gel.

The complementary DNA (cDNA) synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA, USA).

2.7.2 Quantification of transcript levels of pro-inflammatory genes Il1b, Il6, and Tnfa

The transcript levels of interleukin 1 beta (Il1b), interleukin 6 (Il6), and tumor necrosis factor alpha (Tnfa) in the large intestine were determined by real-time polymerase chain reaction (RT-qPCR). The reaction was performed using 2.0µl of cDNA (final concentration of 20ng), 5.0µl of Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA), and 0.2µmol/l (final concentration) of each primer (Table 2), in a final volume of 10µl. The qPCR reactions were performed at 95°C (20s) followed by 40 cycles at 95ºC (3s) and 60°C (30s). The primers used are shown in Table 2. All samples were assayed in triplicate, normalized to β-actin as a housekeeping gene, and the amplification specificity of each amplicon was analyzed using the dissociation curve. The relative quantification of each target gene mRNA level was determined using the $2^{-\Delta\Delta CT}$ method [35].

Table 2. Sequences of primers used for RT-qPCR assay of Il1b, Il6, and Tnfa

Gene		Primers sequence (5'- 3')	Reference
Interleukin 1 beta (Il1b)	F R	CACCTCTCAAGCAGAGCACAG GGGTTCCATGGTGAAGTCAAC	[36]
Interleukin 6 (Il6)	F R	GCCAGAGTCATTCAGAGCAATA GTTGGATGGTCTTGGTCCTTAG	[37]
Tumor necrosis	F	AAATGGGCTCCCTCTCATCAGTTC	[36]

factor alpha (Tnfa)	R	GTCGTAGCAAACCACCAAGCAGA	
β Actin	F	GTCGTACCACTGGCATTGTG	[38]
	R	CTCTCAGCTGTGGTGGTGAA	

F: forward primer; R: reverse primer.

2.8 Statistical analysis

The data obtained from microbial populations and bile acids were analyzed according to a completely randomized 2 × 2 × 2 factorial design, considering two diets (CT and HF) with (+) or without (-) the addition of coffee and two treatment periods (1st and 8th week).

The variables from the other experiments were analyzed according to a completely randomized 2 × 2 factorial design (diets and coffee). Homogeneity of the variances between treatments was assumed and after analysis of variance the significant interactions between the factors were unfolded and compared using the F test and Tukey test. The box plot method was used to remove the outlier and the animals' initial weight was used as a covariate. 0.05 was adopted as a critical probability level for type I errors, using PROC MIXED from SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1 Effect of freeze-dried coffee solution consumption on food intake and body weight

The effect of FCS on food intake and body weight gain was measured in the rats treated with the control or and high-fat diet (Table 3). The addition of FCS to the control (CT+) or high-fat diet (HF+) did not significantly affect (P> 0.05) food intake or

body weight gain. However, the rats fed a high-fat diet (HF-) showed lower (P <0.05) total and daily food intake and higher weight gain compared to the control rats (CT-).

Table 3. Food intake, final body weight, and average daily weight gain of rats treated with control or high-fat diet with or without freeze-dried coffee solution.

Diet	FCS		Mean ± SE	*P*-value		
	(-)	(+)		Diet	FCS	Diet × FCS
	Total Food Intake (g/56 days)			0.001	0.989	0.218
CT	1,020.51 ± 32.95	1,063.63 ± 33.00	1,042.07 ± 23.22[a]			
HF	893.98 ± 35.46	851.76 ± 32.83	872.87 ± 24.16[b]			
Mean	957.25 ± 24.20	957.69 ± 23.26				
	Food Intake (g/d)			0.001	0.990	0.219
CT	18.22 ± 0.59	18.99 ± 0.59	18.61 ± 0.41[a]			
HF	15.96 ± 0.63	15.21 ± 0.59	15.59 ± 0.43[b]			
Mean	17.09 ± 0.43	17.10 ± 0.42				
	FBW (g)			0.004	0.648	0.447
CT	386.20 ± 15.06	390.84 ± 15.14	388.52 ± 10.64[b]			
HF	459.93 ± 15.06	441.31 ± 15.03	450.62 ± 10.64[a]			
Mean	423.07 ± 10.67	416.08 ± 10.67				
	ADG (g/d)			0.004	0.648	0.447
CT	4.80 ± 0.27	4.88 ± 0.27	4.84 ± 0.19[b]			
HF	6.12 ± 0.27	5.78 ± 0.27	5.95 ± 0.19[a]			
Mean	5.46 ± 0.19	5.33 ± 0.19				

Values are means ± S.E., n = 7/group. Means in a column without a common letter differ (*P* <0.05), according to the F test. CT (-): rats treated with control diet AIN-93G; CT (+): rats treated with control diet AIN-93G + FCS; HF (-) rats treated with high-fat diet; HF (+) rats treated with high-fat diet + FCS; FBW = final body weight; ADG = average daily weight gain.

3.2 Hepatic and serum lipid concentration

The hepatic and serum lipid profiles were determined in the rats treated with the control or high-fat diet, with or without the addition of FCS (Table 4). The FCS intake caused a significant (P <0.05) increase in serum HDL concentration, independently of

274 the diet type. The high-fat diet promoted an increase of total cholesterol and
275 triglycerides in the liver compared to the control diet.

276

277 **Table 4. Serum and hepatic lipid profile of rats treated with control or high-fat**
278 **diet with or without freeze-dried coffee solution.**

Diet	FCS		Mean ± SE	*P*-value		
	(-)	(+)		Diet	FCS	Diet × FCS
	CLT serum (mg/dL)			0.562	0.730	0.627
CT	70.22 ± 6.11	71.06 ± 7.53	70.64 ± 5.25			
HF	67.67 ± 9.67	61.63 ± 7.83	64.65 ± 6.81			
Mean	68.94 ± 5.13	66.34 ± 4.96				
	CLT liver (mg/dL)			0.015	0.101	0.409
CT	8.90 ± 2.44	15.14 ± 2.52	12.02 ± 1.83^{b}			
HF	18.57 ± 2.65	20.78 ± 2.37	19.68 ± 1.83^{a}			
Mean	13.74 ± 1.69	17.96 ± 1.69				
	TG serum (mg/dL)			0.869	0.362	0.965
CT	111.20 ± 16.30	96.71 ± 17.85	103.95 ± 12.57			
HF	108.84 ± 17.21	92.90 ± 15.48	100.87 ± 11.78			
Mean	110.02 ± 10.97	94.8 ± 11.69				
	TG liver (mg/dL)			0.038	0.171	0.377
CT	79.34 ± 19.21	123.83 ± 19.82	101.59 ± 14.43^{b}			
HF	146.69 ± 20.91	157.12 ± 18.69	151.91 ± 14.43^{a}			
Mean	113.03 ± 13.31	140.46 ± 13.31				
	HDL serum (mg/dL)			0.863	0.007	0.730
CT	63.62 ± 7.95	86.88 ± 8.15	75.26 ± 5.94			
HF	59.26 ± 8.89	88.06 ± 7.67	73.66 ± 5.94			
Mean	61.44 ± 5.55^{B}	87.47 ± 5.55^{A}				
	HDL liver (mg/dL)			0.415	0.695	0.940
CT	7.53 ± 0.52	7.77 ± 0.54	7.65 ± 0.39			
HF	8.06 ± 0.56	8.23 ± 0.50	8.14 ± 0.39			
Mean	7.80 ± 0.36	8.00 ± 0.36				
	LDL serum (mg/dL)			0.790	0.669	0.358
CT	21.7 ± 3.58	19.87 ± 3.44	20.79 ± 2.57			
HF	17.33 ± 3.69	22.13 ± 3.40	19.73 ± 2.57			
Mean	19.51 ± 2.39	21.00 ± 2.39				
	LDL liver (mg/dL)			0.538	0.743	0.326
CT	4.55 ± 0.64	5.35 ± 0.61	4.95 ± 0.47			
HF	4.69 ± 0.62	4.29 ± 0.64	4.49 ± 0.47			
Mean	4.62 ± 0.42	4.82 ± 0.42				

279 Values are means ± S.E., n = 5/group. CT (-): rats treated with control diet AIN-93G;
280 CT (+): rats treated with control diet AIN-93G + FCS; HF (-) rats treated with high-fat

diet; HF (+) rats treated with high fat diet + FCS. Means on the same line without a common capital letter $^{A, B}$ differ ($P < 0.05$) and means in the same column without a common lowercase letter ^{a,b} differ ($P < 0.05$), according to the F test. CLT = total cholesterol; TG = triglyceride; HDL = high density lipoprotein cholesterol; LDL = low density lipoprotein cholesterol.

3.3 Bile acid concentration in feces

The concentration of bile acids was determined in the feces of the rats treated with the control and high-fat diets, with or without the addition of FCS, at the first and eighth weeks of treatment (Fig. 1). Independently of the presence or absence of FCS in the diets, the concentration of bile acids in the rats' feces was affected by the type of diet and treatment time (weeks). In the first and eighth weeks of the dietary treatment, the fecal concentration of bile acids was significantly ($P < 0.05$) higher in the rats treated with the high-fat diet compared to the control rats. The rats treated with the high-fat di*et al*so showed a significantly ($P < 0.05$) higher concentration of bile acids in their feces in the eighth week of treatment compared to the first week.

Fig 1. Bile acid concentration in feces. Values are least square means of diet x treatment time interaction of bile acid concentration in feces ± S.E., n = 5/group. CT: rats treated with control diet AIN-93G; HF: rats treated with high fat diet. Means without a common capital letter A, B differ ($P < 0.05$) in relation to the effect of diet type on treatment time and means without a common lowercase letter a,b differ ($P < 0.05$) in relation to the effect of treatment time on diet type, according to the F test.

3.4 Determination of fecal microbiota composition

The absolute quantification of specific groups of bacteria was determined in the feces of the rats treated with the control or high fat diet, with or without the addition of FCS (Fig. 2, 3, 4 and 5). The *P*-values of the statical analysis are presented in S1 Table.

The fecal population of *Bacteroides* spp. was the only one that showed a diet x FCS x time interaction effect (Fig. 2). In the first week of treatment, the population of *Bacteroides* spp. in the feces of the rats treated with the high-fat diet (HF-) was significantly ($P < 0.05$) lower compared to the other groups of rats, while the addition of FCS to the high-fat diet (HF+) normalized the fecal *Bacteroides* spp. concentration (CT- x HF+; $P > 0.05$).

In the eighth week of the dietary treatment, there was no significant effect of FCS on the population of *Bacteroides* spp. in the feces of the rats treated with the control or high-fat diet.

Fig 2. *Bacteroides* spp. population in fecal samples. Values are least square means of the diet x FCS x treatment time interaction of *Bacteroides* spp. population in fecal samples of rats treated with control diet without FCS CT (-), control diet + FCS CT (+), high-fat diet HF (-), or high-fat diet + FCS HF (+). Data represent mean log10 16S rRNA gene copies number/g feces. Values are means ± S.E., n = 5/group. Means without a common lowercase letter differ ($P < 0.05$), according to the Tukey test.

The fecal population of *Bifidobacterium* spp. presented a diet x FCS interaction effect (Fig. 3). The addition of FCS to the high-fat diet (HF+) significantly increased ($P < 0.05$) the population of *Bifidobacterium* spp. in the rats' feces compared to the high-fat

diet without FCS (HF-). The addition of FCS to the control diet did not affect (P > 0.05) the population of *Bifidobacterium* spp.

Fig 3. *Bifidobacterium* population in fecal samples Values are least square means of the diet x FCS interaction of *Bifidobacterium* population in fecal samples of rats treated with control diet without FCS CT (-), high-fat diet HF (-), control diet + FCS CT (+), or high-fat diet + FCS HF (+). Data represent mean log10 16S rRNA gene copies number/g feces. Values are means ± S.E., n = 5/group. Means without a common capital letter A,B differ (P <0.05) in relation to the effect of diet type on coffee consumption and means without a common lowercase letter a,b differ (P <0.05) in relation to the effect of coffee consumption on diet type, according to the F test.

The high-fat diet significantly affected (P <0.05) the populations of *Lactobacillus* spp. and *E. coli* in the rats' feces, regardless of the presence of FCS or the treatment time (Fig.4). The *Lactobacillus* spp. population was significantly (P <0.05) higher in the feces of the rats treated with the high-fat diet (HF), while the *E. coli* population was significantly (P <0.05) lower compared to those treated with the control diet.

Fig 1. Populations of *Lactobacillus* and *Escherichia coli* in fecal samples of rats treated with control or high-fat diet. Data represent mean log10 16S rRNA gene copies number/g feces. Values are means ± S.E., n = 5/group. Within the same bacteria strain, columns without a common letter differ, (P < 0.05) according to the F test.

The treatment time (weeks) with the experimental diets significantly affected (P <0.05) the populations of *Lactobacillus* spp., *Enterococcus* spp., and *E. coli*, regardless

of the dietary fat content or the addition of FCS to the diet (Fig. 5). The populations of *Lactobacillus* spp., *Enterococcus* spp., and *E.coli* were significantly ($P < 0.05$) higher in the rats' feces in the first week of treatment with the experimental diets compared to in the eighth week.

Fig 2. Populations of *Lactobacillus* spp., *Escherichia coli,* and *Enterococcus* spp. in fecal samples. Rats are treated with control or high-fat diet, with or without FCS, after 1st and 8th weeks of treatment. Data represent mean log10 16S rRNA gene copies number/g feces. Values are means ± S.E., n = 5/group. Within the same bacteria strain, columns without a common letter differ ($P < 0.05$), according to the F test.

3.5 Microbiota composition of the cecum content

The populations of *Enterococcus* spp., *Bifidobacterium* spp., and *E. coli* present in the rats' cecum contents were significantly affected ($P < 0.05$) by the addition of FCS to the diets (Table 5). The *Enterococcus* spp. population in the cecum content significantly increased ($P < 0.05$) with the addition of FCS to the control diet (CT+) compared to the control di*et al*one (CT-). The population of *Bifidobacterium* spp. significantly increased ($P < 0.05$) when FCS was added to the high-fat diet (HF+) in relation to the high-fat di*et al*one (HF-), however no difference was observed in the *Bifidobacterium* spp. population when FCS was added to the control diet. The *E. coli* population of the cecum content showed a significant increase ($P < 0.05$) when coffee was added to the control diet, while a significant reduction ($P < 0.05$) was observed when FCS was combined with the high-fat diet, compared to the CT- and HF- diets, respectively. The *Enterococcus* spp. population was not significantly affected ($P > 0.05$) by the type of diet in which FCS was incorporated. The high-fat diet (HF-) promoted an

increase in the *Enterococcus* spp. population in the cecum content and did not affect *Bifidobacterium* spp. and *E. coli* compared to the control diet (CT-).

The populations of *Bacteroides* spp. and *Lactobacillus* spp. in the cecum contents were not significantly affected (P >0.05) by FCS consumption, independently of whether the control or high-fat diet was used. However, the high-fat diet promoted a significant increase (P <0.05) in the population of *Lactobacillus* spp. in the rats' cecum content.

Table 5. **Bacterial population of cecum content of rats treated with control or high-fat diet with or without freeze-dried coffee solution.**

Diet	FCS		Mean ± SE	*P*-value		
	(-)	(+)		Diet	FCS	Diet × FCS
	log10 16S rRNA gene copies number/g feces					
	Bacteroides spp.			0.610	0.486	0.784
CT	4.68 ± 0.10	4.78 ± 0.10	4.73 ± 0.07			
HF	4.64 ± 0.11	4.69 ± 0.10	4.67 ± 0.08			
Mean	4.66 ± 0.07	4.73 ±0.07				
	Lactobacillus spp.			0.001	0.338	0.167
CT	3.51 ± 0.14	3.85 ± 0.12	3.68 ± 0.10^{b}			
HF	4.41 ± 0.16	4.33 ± 0.12	4.37 ± 0.10^{a}			
Mean	3.96 ± 0.09	4.09 ± 0.09				
	Enterococcus spp.			0.037	0.194	0.036
CT	0.27 ± 0.14^{Bb}	0.70 ± 0.11^{Aa}	0.49 ± 0.10			
HF	0.93 ± 0.13^{Aa}	0.81 ± 0.11^{Aa}	0.87 ± 0.09			
Mean	0.61 ± 0.08	0.75 ± 0.07				
	Bifidobacterium spp.			0.370	0.001	0.015
CT	1.77 ± 0.25^{Aa}	2.12 ± 0.26^{Aa}	1.94 ± 0.19			
HF	1.33 ± 0.28^{Ba}	3.09 ± 0.24^{Ab}	2.21 ± 0.19			
Mean	1.55 ± 0.17	2.61 ± 0.17				
	Escherichia coli			0.003	0.263	0.003
CT	1.82 ± 0.20^{Ba}	3.02 ± 0.21^{Aa}	2.42 ± 0.15			
HF	1.73 ± 0.22^{Aa}	0.98 ± 0.19^{Bb}	1.36 ± 0.15			
Mean	1.78 ± 0.13	2.00 ± 0.13				

Values are means ± S.E., n = 5/group. CT (-): rats treated with control diet AIN-93G; CT (+): rats treated with control diet AIN-93G + FCS; HF (-) rats treated with high-fat diet; HF (+) rats treated with high-fat

diet + FCS. Means on the same line without a common capital letter ^{A,B} differ ($P < 0.05$) and means in the same column without a common lowercase letter ^{a,b} differ ($P < 0.05$), according to the F test.

3.6 Inflammatory genes in the large intestine

The mRNA levels of Il1b, Il6, and Tnfa were evaluated in the rats treated with the control and high-fat diets, with and without FCS (Table 6). The addition of FCS to the high-fat diet (HF+) decreased ($P < 0.05$) the Il1b mRNA levels in the large intestine compared to the high-fat di*et al*one (HF-), while no difference was observed between the CT- and CT+ groups. Regarding the Il6 mRNA levels in the large intestine, FCS increased these regardless of the diet type, while the high-fat diet promoted a decrease, independently of whether or not FCS was used. The Tnfa mRNA levels in the large intestine were not significantly affected ($P > 0.05$) by the diet type or presence of FCS.

Table 6. **Quantification of interleukin-1beta (Il1b), interleukin-6 (Il6), and tumor necrosis factor alpha (Tnfa) mRNA levels in the large intestine of rats treated with control or high fat diet with or without freeze-dried coffee solution.**

Diet	Coffee		Mean ± SE	*P*-value		
	(-)	(+)		Diet	Coffee	Diet × Coffee
	mRNA Il1b			0.068	0.049	0.006
CT	1.00 ± 0.09 ^{Aa}	1.19 ± 0.09 ^{Aa}	1.10 ± 0.06			
HF	1.21 ± 0.10 ^{Aa}	0.63 ± 0.10 ^{Bb}	0.91 ± 0.07			
Mean	1.11 ± 0.06	0.91 ± 0.06				
	mRNA Il6			0.025	0.018	0.825
CT	1.02 ± 0.23	1.62 ± 0.21	1.32 ± 0.16^{a}			
HF	0.53 ± 0.19	1.04 ± 0.21	0.90 ± 0.14^{b}			
Mean	0.78 ± 0.15^{B}	1.33 ± 0.15^{A}				
	Tnfa			0.849	0.580	0.287
CT	0.97 ± 0.22	0.87 ± 0.22	0.92 ± 0.15			
HF	0.72 ± 0.18	1.05 ± 0.18	0.88 ± 0.13			
Mean	0.85 ± 0.14	0.96 ± 0.14				

Values are means ± S.E., n = 6/group. CT (-): rats treated with control diet AIN-93G; CT (+): rats treated with control diet AIN-93G + FCS; HF (-) rats treated with high-fat diet; HF (+) rats treated with high-fat

diet + FCS. Means on the same line without a common capital letter^{A,B} differ ($P < 0.05$) and means in the same column without a common lowercase letter^{a,b} differ ($P < 0.05$), according to the F test.

4. Discussion

This study evaluated the effect of FCS when mixed with the diet on the physiological parameters, lipid profile, bile acids, and microbiota of rats in a high-fat diet-induced obesity model.

Several studies have shown the effectiveness of coffee extract in reducing body weight gain in rats treated with a high-fat diet [39–44]. Polyphenols, which are present in a high concentration in coffee, are able to inhibit digestive enzymes [45] and consequently inhibit macronutrient absorption and reduce body weight gain [16,20]. Contrary to what has been observed in some studies, in the present study the consumption of FCS for 56 days did not change body weight in the control group or high-fat diet rats. This result suggests that in a treatment model for preventing obesity, when FCS is co-administrated during the development of obesity, a longer treatment time may be necessary to observe the effect of FCS on body weight. A previous study that monitored obesity induction over 83 days by a high-fat diet showed that although the rats on the high-fat diet had a higher body weight than the control rats after day 21 of the treatment, statistical significance was reached only after 83 days [46]. This reinforces the hypothesis that in the present study the rats were in a pre-obesity state. Furthermore, several studies that have observed significant effects of coffee on body weight [39,41,43] have used an obesity treatment model instead of an obesity prevention one as in the present study.

The higher body weight of the rats treated with the high-fat diet despite their lower food intake is associated with the higher energy density of the high-fat diet (5.30

kcal/g) compared to the control (3.95 kcal/g). Even though the dietary intake of rats treated with the high-fat diet was 16% less than the control rats, their energy intake was 13% greater. Similar results have been observed in other studies [47–49]. The authors argue that the decrease in food intake promoted by the high-fat diet may be attributed to a compensatory mechanism that attempts to maintain energy balance homeostasis and consequently control the excessive body weight gain induced by the high caloric density of the diet.

Several other factors, such as coffee bean species (*Coffeea arabica* and *Coffea canephora* var. *Robusta*), roasting process (time x temperature) [50], and the method used to prepare the beverage, influence coffee polyphenol content. Rendon *et al*. [51] showed that filtered coffee beverages have lower diterpene contents than unfiltered ones. According to Cruz *et al*. [52], variations in the biological activity of coffee may be related to such differences in the chemical composition of the beverage.

In the present study, the coffee solution was freeze-dried to enable its incorporation into the high-fat diet. Although this process may promote chemical alterations in the polyphenols, such modifications can be considered minimal, as freeze drying is the most recommended method for preserving polyphenol compounds [53,54]. Another aspect to be considered is the dose of FCS used in the present study (0.39g/100g of diet), which is equivalent to 163 ml/day or 1.5 cups/day of coffee, the usual mean coffee intake of the Brazilian population [21]. When testing two doses of coffee extract (40 and 60% v/v), Maki *et al*. [55] only observed a significant reduction in body weight gain and the amelioration of some biochemical markers in the mice treated with the high dose of 60% coffee extract. Therefore, it is possible that the dose used in the present study was insufficient to reduce the rats' body weight.

Regarding lipide profile, there is no consensus concerning the effect of coffee [43,56–58]. In the present study, the consumption of FCS did not affect most of the lipid profile markers in the serum and liver, since the cholesterol, triglyceride, and LDL levels were similar between the control and high-fat diets, with and without the addition of FCS. Similar to our results, Karabudak *et al.* [59] demonstrated no significant association between Turkish or instant coffee consumption in subjects. The authors suggested that the coffee preparation method and the amount consumed are important aspects in serum lipid response. The cause of differences is not clear but may be associated with the amount of coffee administrated, different routes of coffee administration (gavage or mixed with the diet), and different treatment protocols, as some studies have employed an obesity treatment protocol rather than an obesity prevention one. Ilmiawati [44] *et al.* observed a decrease in serum cholesterol and triglycerides with low doses of a green coffee extract (GCE), however only the high doses of GCE (> 20 mg/kg BWG/d) were able to decrease LDL and increase HDL in serum.

Although FCS did not decrease total cholesterol or the atherogenic LDL lipoprotein, coffee consumption did promote a significant increase in serum HDL lipoproteins. This result shows that coffee has atheroprotective properties, as HDL lipoproteins remove excess cholesterol by reverse cholesterol transport to tissues [60]. This is also in line with the marginal increase in total cholesterol in the liver observed with the coffee treatment (1.31-fold compared to no coffee; $p = 0.100$). Similar to our result, Feyisa *et al.* [56] observed that rats treated with coffee had higher HDL-c concentrations. Some studies, most of which use purified compounds, show that some polyphenols found in coffee (caffeic, ferulic, and phenolic acids), as well as in other

foods, stimulate reverse cholesterol transport by promoting HDL formation and cholesterol efflux [61,62].

Contrary to what was expected, in the rats treated with the high-fat diet no change was observed in serum lipid profiles, and only the hepatic concentration of cholesterol and triglycerides increased. We hypothesize that the obesity induction time was not enough to surpass systemic homeostasis, therefore excess cholesterol was taken up by the liver through LDL receptors mediated by the SCAP-SREBP pathway [63], which caused an increase in hepatic cholesterol, while serum levels remained similar to those of the control rats. The higher bile acid excretion in stools observed in the first week of treatment, which was even greater in the eighth week, in the rats treated with the high-fat diet regardless of FCS consumption, reinforces the hypothesis that systemic homeostasis was maintained until 56 days of treatment.

Bile acids are amphipathic sterols secreted in the duodenum. They are the main constituent of bile and are responsible for emulsifying fat and facilitating digestion and absorption [64]. As expected, the high-fat diet promoted an increase of total bile acids in the rats' feces. Lin *et al*. [11] found lower concentrations of conjugated bile acids and higher levels of unconjugated bile acids in the feces of rats treated with a high-fat diet. Gut microbiota is capable of hydrolyzing conjugated bile acids into secondary bile acids, which are associated with colon cancer [65,66]. Although coffee and its polyphenolic compounds can decrease dietary lipid digestion by reducing the synthesis and action of bile acids [67,68], in the present study the FCS did not affect total bile acid excretion in the feces.

Certain strains of the *Bifidobacterium* genus engage in anti-obesity activity and are therefore found in lower populations in obese individuals. The increase in *Bifidobacterium* spp. in the feces and cecal content promoted by the FCS only in the

rats treated with the high-fat diet suggests that the consumption of FCS during the process of obesity development and progression may have a protective effect, since these rats also presented lower mRNA levels of the pro-inflammatory Il1b in the large intestine. These effects may be associated with the high polyphenol content of coffee (chlorogenic acids, caffeine, cafestol, kahweol), which present anti-inflammatory properties [18,69]. Some studies have shown that polyphenols in foods have prebiotic effects, favoring the *Bifidobacterium* spp. population in the gut [70,71]. Fermentation products from coffee components may inhibit the growth of other microorganisms in the colon, and consequently favor an increase in the *Bifidobacterium* genus in rats treated with coffee combined with a high-fat diet. Nakayama and Oishi [31] showed an increase in *Bifidobacterium* spp. in the feces of rats treated with coffee extract, thus corroborating with our results.

Although the *Lactobacillus* genus is highly associated with host health benefits, some subspecies appear to be positively associated with body weight gain [8]. The high body weight gain observed in the rats treated with the high-fat diet in the present study may be associated with some subspecies of the *Lactobacillus* population generally observed in obese organisms, since a significant increase in *Lactobacillus* spp. was observed in the feces of these rats. Similar to our results, Cowan *et al*. [39] did not observe any effect of coffee administration on the fecal population of *Lactobacillus* spp.

E. coli is generally found in the human gastrointestinal tract, either as commensal, probiotic, or pathogen bacteria [72], comprising less than 0.1% of total bacterial cells in the gut microbiota [73]. The present study observed a reduction in fecal *E. coli* populations with the high-fat diet treatment, while no FCS effect was observed. Corroborating our findings, Million *et al*. [74] suggested that the absence of *E. coli* was an independent predictor of weight gain, as they found *E. coli* in the feces of

patients with weight loss. In vitro studies have shown that phenolic compound extract modulates *E. coli* growth [75–77]. Therefore, in the present study coffee polyphenols or the products of their hydrolization or reduction may have stimulated the growth of *E. coli* in the cecum of the rats fed with the control diet. In line with data from the literature, *Lactobacillus* spp., *E. coli*, and *Enterococcus* spp. populations decreased in the feces with increased treatment time, independent of the dietary treatment employed. Given the wide density and diversity of microorganisms in the gastrointestinal tract, especially in the colon, the release/production of different second metabolites by these microorganisms (organic acids and short chain fatty acids) may explain the reduction of some bacterial strains, since these compounds act as antimicrobial agents [6,78]. Therefore, the longer dietary treatment time may have increased the antimicrobial action of some metabolites on *Lactobacillus*, *E. coli*, and *Enterococcus*, reducing these bacterial populations in the feces.

Lachnospira, *Roseburia*, *Butyrivibrio*, *Ruminoccus*, *Fecalibacterium*, and *Fusobacteria* constitute the main microorganisms in the cecum [79]. However, in the present study, *E. coli*, *Enterococcus* spp., *Lactobacillus* spp., *Bacteroides* spp., and *Bifidobacterium* spp. were identified in the cecum. According to Liu *et al*. [16], *Enterococcus* and *Bifidobacterium* hydrolyze food polyphenols. Therefore, the observed increase in *Bifidobacterium* and *Enterococcus* in the cecum of the rats treated with FCS combined with the control diet and FCS combined with the high-fat diet compared to the control and high-fat diets alone, respectively, may be associated with polyphenols from coffee. In addition to the effect of FCS, it is likely that some fecal strains of *Bifidobacterium* present mechanisms of resistance to bile acids, which results in an increase in its population. In some strains, such as *Bifidobacterium logum* and

Bifidobacterium brevis, mechanisms of resistance to bile acids involving bile efflux systems have already been identified [80–82].

Considering that pro-inflammatory cytokines such as Il6, Il1b, and Tnfa are increased in obese individuals [83] and that consuming four or more cups of coffee/day seems to decrease serum Il6 levels [84], in our study we determined the mRNA levels in the rats' large intestine in order to investigated a possible coffee x fecal microbiota x gut inflammatory response interaction. Despite the high polyphenol content in coffee, the Il6 mRNA levels were increased by coffee consumption regardless of the diet type, while the high-fat diet decreased Il6 mRNA levels in the large intestine. The decrease in Il6 mRNA levels in the large intestine promoted by the high-fat diet in the rats may be related to the inhibition of the *E. coli* population observed in these rats compared to the control. According to Kittana *et al*. [85], a greater population of commensal *E. coli* has been observed in the intestinal tissue of humans during the inflammation process. These authors also demonstrated that some strains of *E. coli* in the intestine are associated with high levels of Il6 secretion.

Despite the accepted idea that foods rich in polyphenols inhibit pro-inflammatory cytokine secretion [86], in the present study coffee promoted an increase in Il6 mRNA levels in the large intestine regardless of the diet type. This contradiction may be attributed to differences in tissue response. Most studies evaluate systemic inflammation by measuring serum levels, while in the present study we evaluated inflammatory response in the large intestine. According to Juge-Aubry *et al*. [87], Il6 may engage in anti-inflammatory activity, since it is able to decrease Tnfa and IFNγ (interferon gamma) over the course of inflammation, in an attempt to control the inflammation and inhibit tissue damage. Caro-Gómez *et al*. [88] observed increased expression levels of hepatic Il6 in rats treated with green coffee extract. The authors

mention that Il6 is important to control obesity-associated inflammation by favoring macrophage polarization towards the M2 phenotype, which acts in the resolution phase of inflammation and in repairing damaged tissues.

Conclusions

The results suggest that the consumption of FCS may promote positive health effect in rats fed with a high-fat diet by increasing the populations of *Bifidobacterium*, improving HDL-c reverse cholesterol transport to tissues, and reducing Il1b mRNA levels. However, FCS administered in a dose equivalent to the usual mean coffee intake of the Brazilian population over an eight-weeks period was not effective in controlling food intake and consequently preventing weight gain in rats during obesity induction by the consumption of a high-fat diet.

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Supporting information

S1 Table. *P*-values of the statical analysis of microbial composition and bile acid concentration in feces of rats fed control diet without FCS CT (-), control diet + FCS CT (+), high fat diet HF (-) or high fat diet + FCS (HF +). D=diet; FCS= freeze-dried coffee solution; T= time; SEM= Standard error of the mean.

Fig1

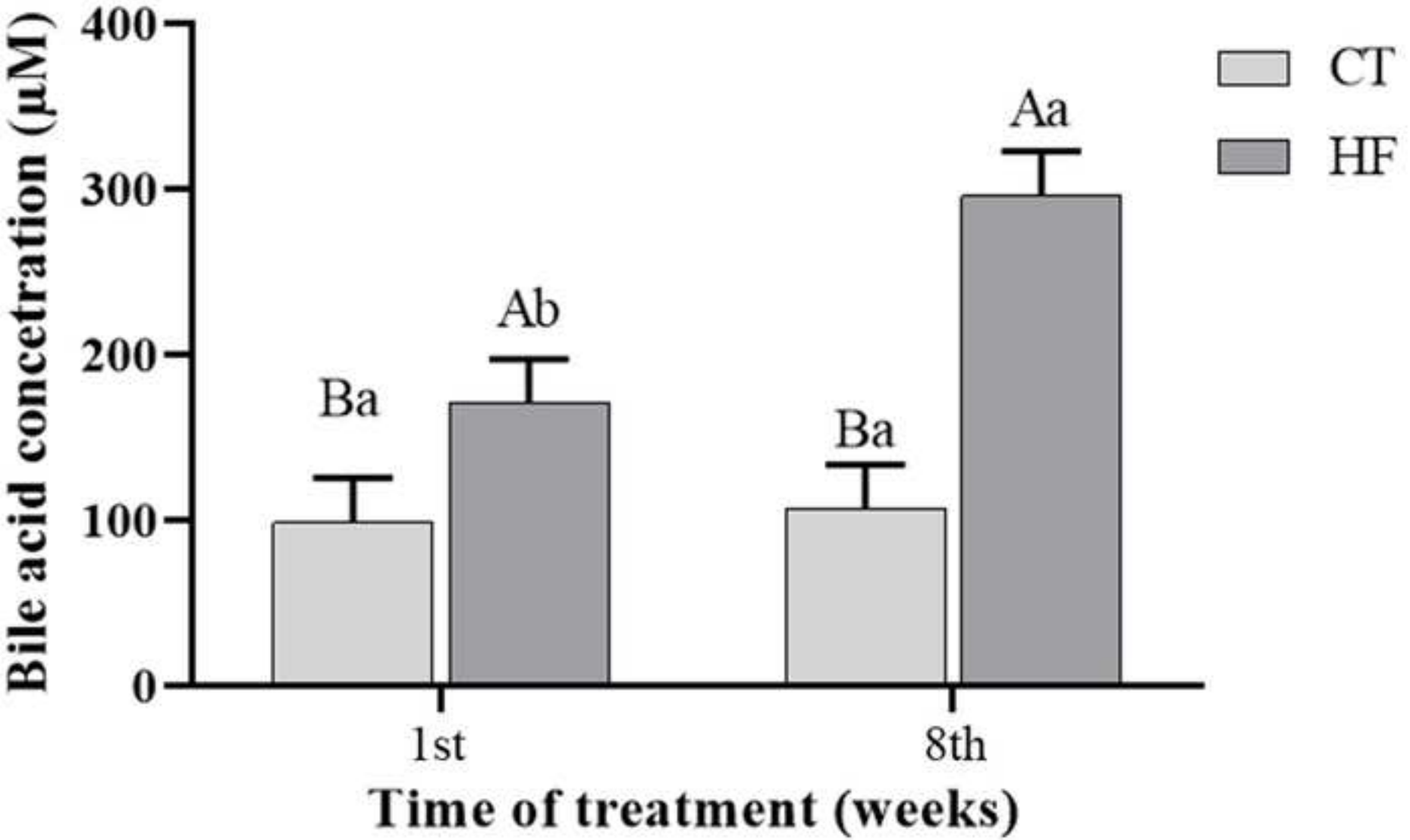

Fig2

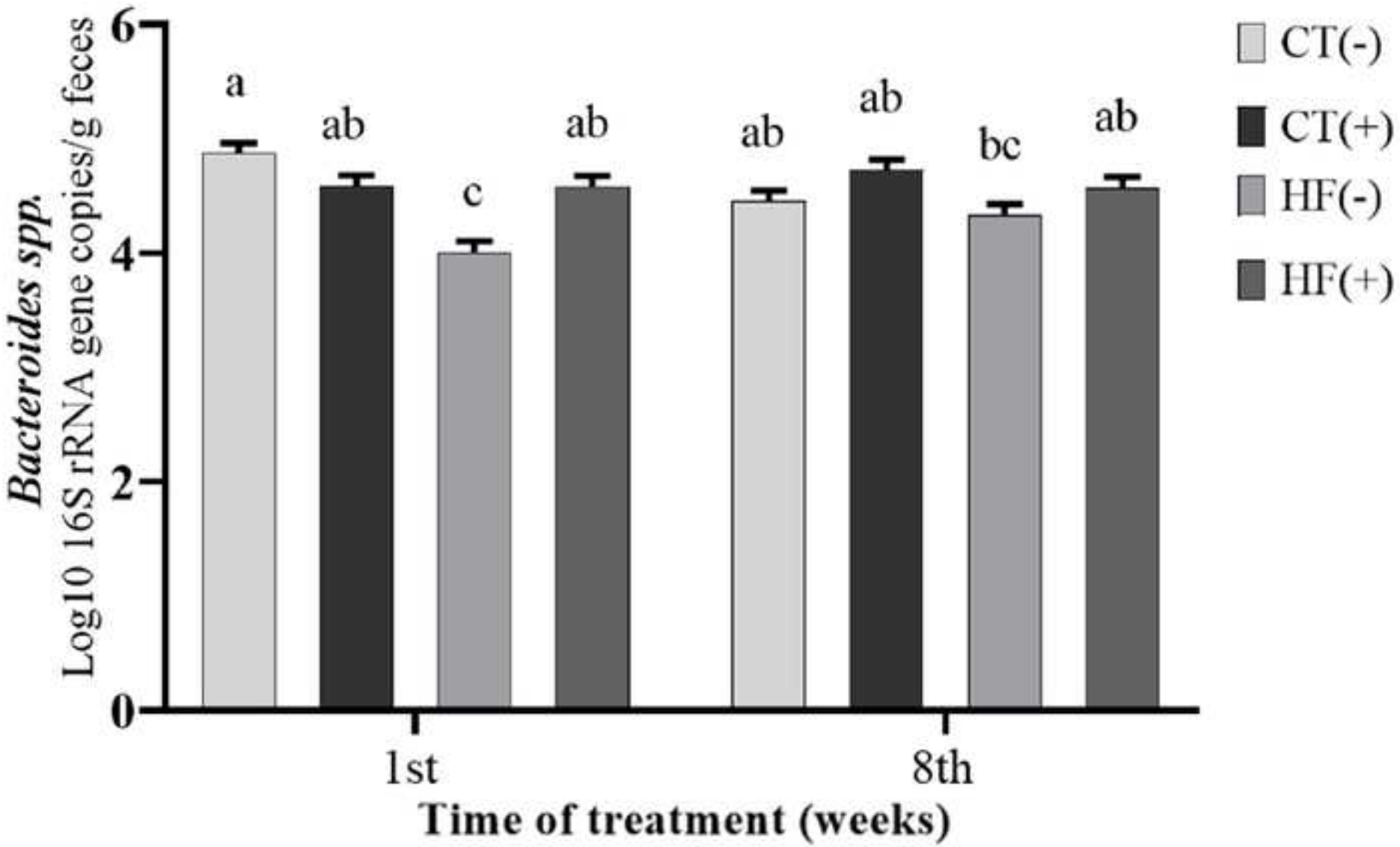

Fig3

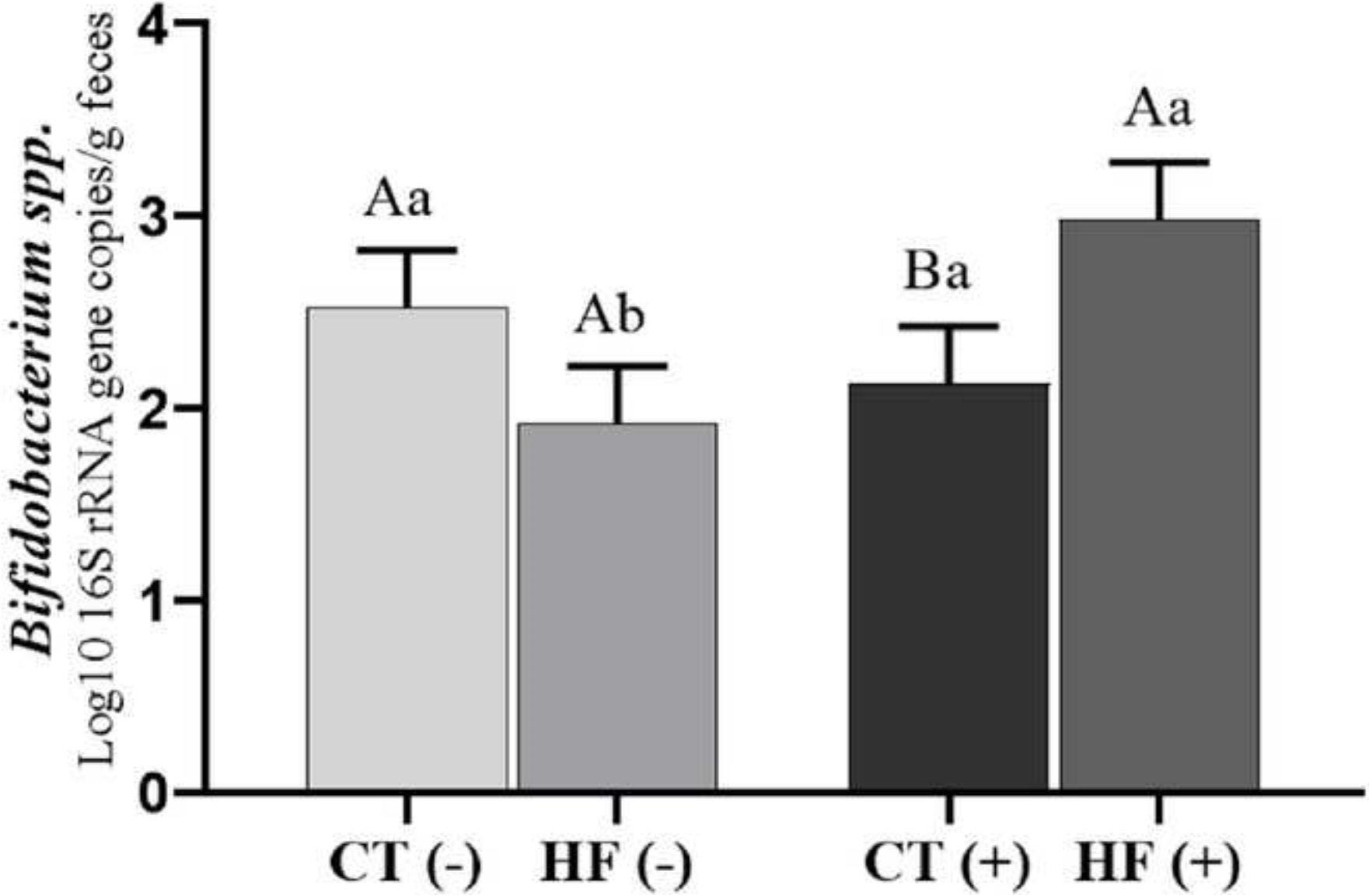

Fig4

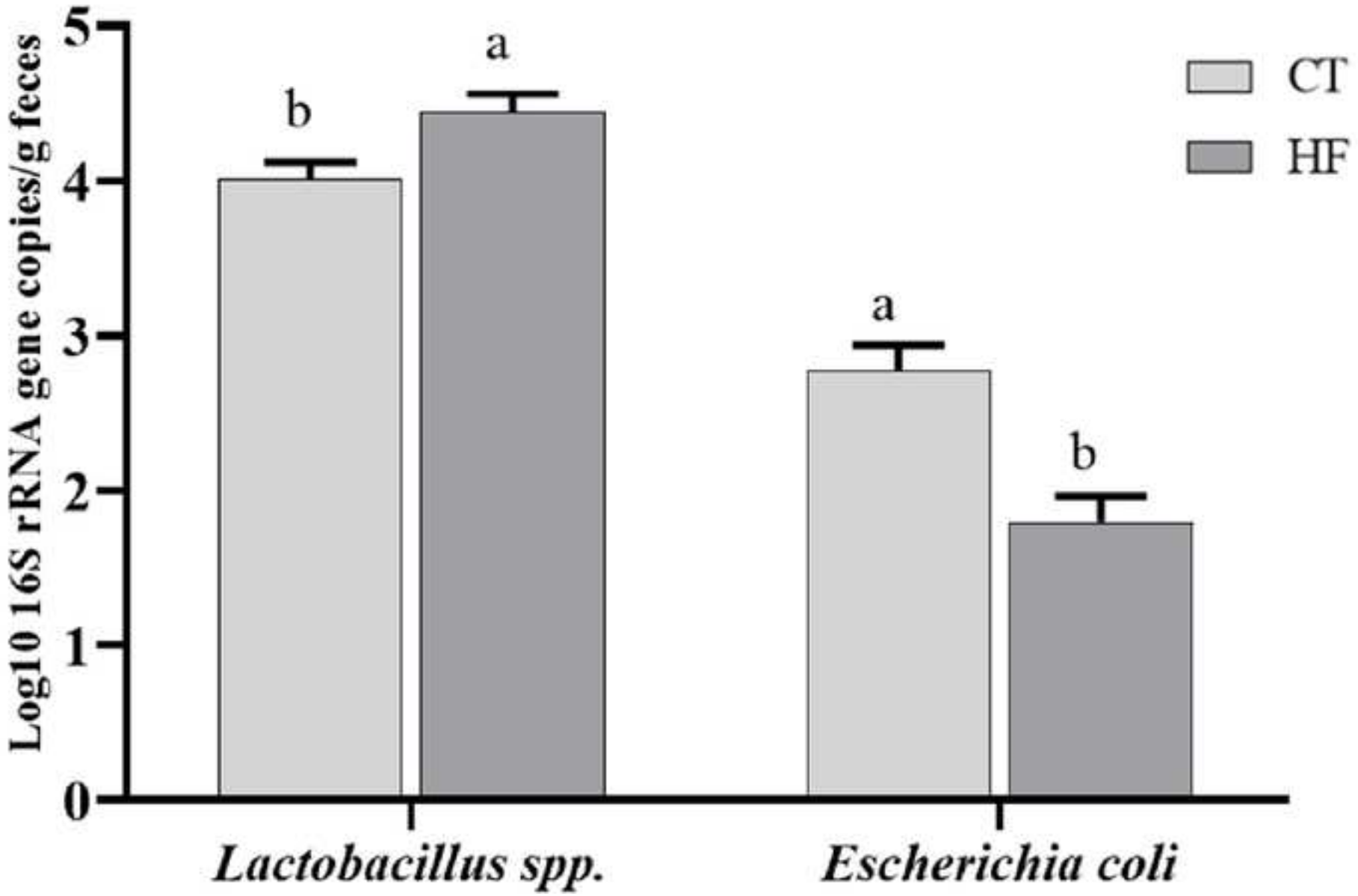

Fig5